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Epigenetic modification of *Rta* (ORF50) promoter is not responsible for distinct reactivation patterns of murine gammaherpesviruses

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Summary. – Gammaherpesviruses-encoded replication and transcription activator (*Rta*) (ORF50) plays an essential role in the initiation of viral lytic gene expression and reactivation from latency. The *Rta* expression is influenced by many viral and cellular factors, including epigenetic modifications, mainly DNA methylation and histone modifications. Murine gammaherpesvirus 68 (MHV-68), belonging to the species *Murid herpesvirus* (MuHV-4), is widely used as a model to study human gammaherpesvirus infections *in vitro* as well as *in vivo*. Recent studies of the MHV-68 *Rta* promoter revealed the effect of DNA demethylation and histone acetylation, induced by the inhibitor of histone deacetylase trichostatin A (TSA), on the MHV-68 reactivation from latency. Two other strains of MuHV-4, MHV-72 and MHV-4556, possess several unique properties, which distinguish them from strain MHV-68. Recently discovered reduced capacity of MHV-72 and MHV-4556 to reactivate from latency may be related to different methylation/demethylation patterns of the promoter regulatory region of the *Rta*. Here, we investigated the epigenetic regulation of the *Rta* promoter of three murine gammaherpesvirus strains, MHV-68, MHV-72 and MHV-4556, during latency and reactivation *in vivo*. However, we did not find any differences between *Rta* of MHV-68, MHV-72 and MHV-4556 and its epigenetic regulation during lytic infection, latency and *de novo* infection after *ex vivo* and *in vivo* reactivation induced by TSA. We confirmed that the treatment with TSA successfully induced demethylation of the *Rta* promoter regions of all three studied strains. Moreover, we have shown that the primary sequence of *Rta* and its promoter is identical for all three strains.

Keywords: murine gammaherpesvirus; trichostatin A; epigenetic modification; histone acetylation; gammaherpesvirus reactivation; replication and transcription activator

Introduction

Members of the subfamily *Gammaherpesvirinae* are relatively widespread viruses that can infect a variety of mammalian species, including humans. Human gammaherpesviruses, Epstein-Barr virus (EBV, HHV-4) and Kaposi´s sarcoma associated herpesvirus (KSHV, HHV-8) are oncogenic viruses that induce a readily controlled lytic infection followed by the establishment of life-long latency (Pagano, 1999; Sarid *et al.,* 1999). In most cases the persistent infection is asymptomatic or accompanied by benign cellular proliferation. However, persistent/chronic gammaherpesvirus infection is occasionally associated with the development of malignancies, such as Burkitt´s lymphoma, Hodgkin´s disease, and nasopharyngeal carcinoma as a result of EBV infection, and Kaposi´s sarcoma, primary effusion lymphoma, and Castleman´s disease after KSHV infection (Barton *et al.,* 2011). Most of our knowledge of latent or acute infection with human gammaherpesviruses

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Abbreviations: BGS = bisulfite sequencing analysis; COBRA = Combined bisulfite restriction analysis; d.p.i. = days post infection; EBV = Epstein-Barr virus; HDAC = histone deacetylases; KSHV = Kaposi´s sarcoma associated herpesvirus; MHV-68 = murine gammaherpesvirus 68; ORF = open reading frame; *Rta* = replication and transcription activator; TSA = trichostatin A

have been derived from *in vitro* studies. However, this approach does not allow revealing a whole complex of virus/host interactions which take place during *in vivo* infection.

Murine gammaherpesvirus 68 (MHV-68), a natural pathogen of murid rodents belonging to the species *Murid herpesvirus 4* (MuHV-4), is widely used as a model to study human gammaherpesviruses, answering recent aspects of virus infection and reactivation from latency, including a chronic infection which is associated with risk of lymphoproliferative disorders and oncogenesis. Together eight murine gammaherpesviruses designated -60, -68, -72, -76, -78, -4556, -5682, and -Šumava were isolated in Slovakia and thereafter in the Czech Republic (Blaškovič *et al.,* 1980; Kožuch *et al.*, 1993). Among them, the best-characterized isolate is MHV-68 (Nash *et al.*, 2001). Despite the fact that the full genome sequence of MHV-72 and MHV-4556 is not known yet, some *in vitro* and *in vivo* pathogenetic studies revealed their close relation to MHV-68. However, several differences were observed among these three strains. It was shown that chronic infection with MHV-72 results in higher incidence of lymphomas in liver, lung and adrenals, which rises to nearly three-fold in mice treated with immunosuppressive drug compared to MHV-68 (Mistríková *et al.*, 1996, 1999). Moreover, replication of MHV-72 in mammary glands and its transmission from infected mother mouse to neonates via breast milk was described. Further differences were revealed in studies focused on characterization of genome restriction map, nucleotide sequences of some unique genes including thymidine kinase, M3 protein, MK3 protein and glycoprotein 150, and some biological properties of MHV-72 and MHV-4556 (Mačáková *et al.,* 2003; Valovičová *et al.*, 2006; Belvončíková *et al.,* 2008; Halásová *et al.*, 2011; Kúdelová *et al.,* 2012). Recent results obtained in our study on biological properties of MHV-72 and MHV-4556 *in vivo* showed reduced ability of these strains to reactivate *ex vivo* from latency in spleen, lungs and thymus treated with trichostatin A (TSA), compared to MHV-68 (Lopušná *et al.*, unpublished data).

All gammaherpesviruses encode a replication and transcription activator (*Rta*) homologue, which plays an essential role in the initiation of viral lytic gene expression and thus reactivation from latency. Two multifunctional proteins in EBV, known as *ZEBRA* (BZLF1) and *Rta* (BRLF1), and the one in KSHV, known as *Rta* (ORF50), are responsible for regulation of latent/lytic switch. The *Rta* encoded by gene ORF50 of gammaherpesviruses shares similarities in genomic location, sequence, and splicing pattern, confirming that they play conserved and important role in virus life cycle (Quinlivan *et al.,* 1993; Ragoczy *et al.,* 1998; Ragoczy and Miller, 2001; Russo *et al.,* 1996; Whitehouse *et al.,* 1997).

MHV-68 *Rta* is responsible for transactivation of both its own promoter and promoters of other viral and cellular genes, resulting in reactivation of latent virus and lytic replication (Hair *et al.,* 2007). It has been previously shown that an *Rta* null mutant is incapable of viral protein synthesis, viral DNA replication or virion production. In addition to the transactivation of viral promoters by *Rta*, the reactivation as well as the phase of viral dormancy are influenced by several cellular factors, including the epigenetic modifications, which have been extensively studied in past ten years. Epigenetic modifications, as changes in gene activity and expression that occur without alteration in DNA sequence, are tightly regulated by two major epigenetic modifications: DNA methylation and histone modifications (Goldberg *et al.,* 2007; Bernstein *et al.,* 2007).

Previous studies have shown that both DNA methylation and histone acetylation regulate EBV and KSHV reactivation from latency (Chen *et al.,* 2001; Lu *et al.,* 2003; Chang and Liu, 2000; Countryman *et al.,* 2008; Ben-Sasson and Klein, 1981). Recently, Yang *et al.* (2009) investigated the effect of DNA demethylation, mediated by methyltransferase inhibitor 5-aza-2-deoxycytidine (5-AzaC), and histone acetylation, induced by the inhibitor of histone deacetylases trichostatin A, on the MHV-68 reactivation from latency. For the effective reactivation of MHV-68 from latency only the histone acetylation induced by TSA was needed. In addition, they identified 15 methylation sites (CpG) localised in the MHV-68 *Rta* promoter. Further analyses confirmed differential methylation patterns of *Rta* promoter in virion, during lytic infection of BHK-21 cells, during latency in S11E cell line transformed by MHV-68 and finally during reactivation induced by 5-aza-2-deoxycytidine and TSA *in vitro* and *in vivo*.

Despite the intensive study, the role of epigenetic regulation in MHV-68, MHV-72 and/or MHV-4556 reactivation *in vivo* has not been reported yet. We suppose that unique properties of MHV-72 and MHV-4556, mainly in the context of reduced capacity to reactivate from latency, may be related to different methylation/demethylation pattern of the promoter regulatory region of the transactivator protein *Rta*. In this study, we investigated the epigenetic regulation of the *Rta* promoter of three murine gammaherpesvirus strains, MHV-68, MHV-72 and MHV-4556 during latency and reactivation.

Materials and Methods

Cells and viruses. BHK-21[C-13] (baby hamster kidney fibroblast cell line, ATCC® CCL-10™) and S11E (murine B-cell line transformed with MHV-68 obtained from prof. J.P. Stewart, PhD., Department of Veterinary Pathology, The University of Edinburgh, United Kingdom) (Usherwood *et al.*, 1996) cells were cultured in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal bovine serum (HyClone), 2 mmol/l L-glutamine, 100 U of streptomycin per ml and 100 U of penicillin per ml (Lonza).

Experiments were performed with the gammaherpesviruses MHV-68 clone f2.6, MHV-72 clone h3.7 (Rašlová *et al.,* 2000) and MHV-4556 clone i2.8 (Valovičová *et al.,* 2006). Working virus stocks were prepared after infection of BHK-21 cells at the multiplicity of infection (MOI) of 0.05 PFU/cell.

Animals. Six-week-old BALB/c mice were obtained from Laboratory Animal Breeding and Experimental Facility of Faculty of Medicine, Masaryk University in Brno, Czech Republic. Mice were bred and housed in a specific pathogen-free barriers facility in accordance with institutional and state guidelines (Ro 3497/14-221). The mice were infected intranasally with 2 x 10^5 PFU of virus in 25 μl of DMEM following an Isoflurane anesthesia. For *ex vivo* reactivation, mice were sacrificed with an Isoflurane overdose followed by cervical dislocation at 14 and 35 days post infection (d.p.i.). Spleens were collected and cultured in DMEM supplemented with TSA (200 ng/ml) for 10 days followed by DNA isolation. For *in vivo* reactivation, mice (35 d.p.i.) were stimulated with TSA (2.4 mg/kg) subcutaneously for 5 hr and subsequently sacrificed with an Isoflurane overdose followed by cervical dislocation. Control mice were handled with the same manner as infected mice with the exception of TSA treatment. Non-infected mice served as a negative control. Spleens were harvested and rapid frozen in liquid nitrogen (-195.79°C) prior to the DNA isolation.

DNA isolation. DNA from S11E cells, infected BHK-21 cells, and spleen after *ex vivo* and *in vivo* reactivation was extracted using the QIAamp DNA mini kit (Qiagen) according to the instructions of the manufacturer. DNA was stored at -20°C until further use.

Bisulfite conversion, COBRA, methylation specific PCR and cloning. Bisulfite conversion of DNA (up to 600 ng) was performed using the EpiJET kit for bisulfite conversion (Thermo Scientific) according to the instructions of the manufacturer. Converted as well as non-converted DNA was analyzed by Combined bisulfite restriction analysis (COBRA) – cleavage combination of methylation specific restriction enzymes *Msp*I/*Hpa*II (Thermo Scientific). *Rta* promoter region, which was divided into two fragments, P1 (65695–66258nt) and P2 (66355–66879nt), was analyzed for methylation status during latency and *in vivo* induced reactivation of MHV-68, MHV-72 and MHV-4556 by methylation specific PCR. Bisulfide converted DNA from spleen of infected mice was amplified by methylation specific and non-specific primers (Table 1). The amplified fragments were purified by Wizard® SV gel and PCR clean-up system (Promega) according to the instructions of the manufacturer. The amplicons were cloned into the pJET1.2 blunt cloning vector system (Thermo Scientific) according to the instructions of the manufacturer and analyzed by sequencing. Obtained sequences were compared with the reference sequence of MHV-68 (Acc. No. U 97553).

Sequencing. For analysis of primary structure of the genomic region corresponding to the ORF50 (66760–69373nt) and the *Rta* promoter region (65644–66800nt) of MHV-72 and MHV-4556 we used conventional PCR with primers listed in Table 1. All fragments were cloned into the pJET1.2 blunt cloning vector system (Thermo Scientific) and analyzed by sequencing. The obtained sequences were compared with the MHV-68 sequence.

Results and Discussion

MHV-68 encodes an immediate-early replication transactivator protein *Rta* (ORF50) in a similar genomic location to other gammaherpesviruses. Moreover, all gammaherpesvirus *Rta* transcripts share a similar architecture. They comprise two exons separated by an intron, which is essentially composed of the ORF49 gene (Fig. 1a,b,c). Splicing of the two exons and

Table 1. List of primers

Name of primer	Sequence (5'- 3')	Genomic location	Notes
CPORF50/ F1	CAAGGACCATGGCAACTAGTCTC	65643-65665	used for sequencing
CPORF50/R2	CTACCTTATCGGCTGAAGGGG	66799-66779	used for sequencing
CPORF50R3	TAGCACATCTGTTGTGTGGGC	66936-66916	used for sequencing
50WF1*1	GGCCGCAGACATTTAATGAC	68147-68166	used for sequencing
50WR1*1	AACTGGAACTCTTCTGTGGC	68733-68714	used for sequencing
ORF50MHVF1	CAAAGTCCATAACAGGCATCC	67939-67959	used for sequencing
ORF50MHVR1	CTGTGGGAGAAGCAAACAGG	68450-68431	used for sequencing
YTHHP1-F*2	GGTTTTTGTGTAGAATTTTTGATTATGA	65695-65722	used for BGS
YTHHP1-R*2	CCAACCTCACCAACTTTTACAATA	66258-66235	used for BGS
YTHHP2-F*2	TTTTTTGAATAGAGTGAGAAGGGTAG	66355-66380	used for BGS
YTHHP2-R*2	TCAAACTAATAACAACACTTTAATTTTTAA	66879-66858	used for BGS
YTHHP1-M-F*2	TGTTGGTTACGTTTAGGTATTCGA	65791-65814	methylation specific, used for BGS
YTHHP1-M-R*2	ATCTCACTAAAAACACTCCAACGAC	66084-66060	methylation specific, used for BGS
YTHHP2-M-F*2	GTATTACGAGGGAATTTTTGTAGC	66753-66776	methylation specific, used for BGS
YTHHP2-M-R*2	ATTTTTAATAAAATACTAATCTATCTACGT	66857-66828	methylation specific, used for BGS

*1Weck *et al.* (1999); *2Yang *et al.* (2009).

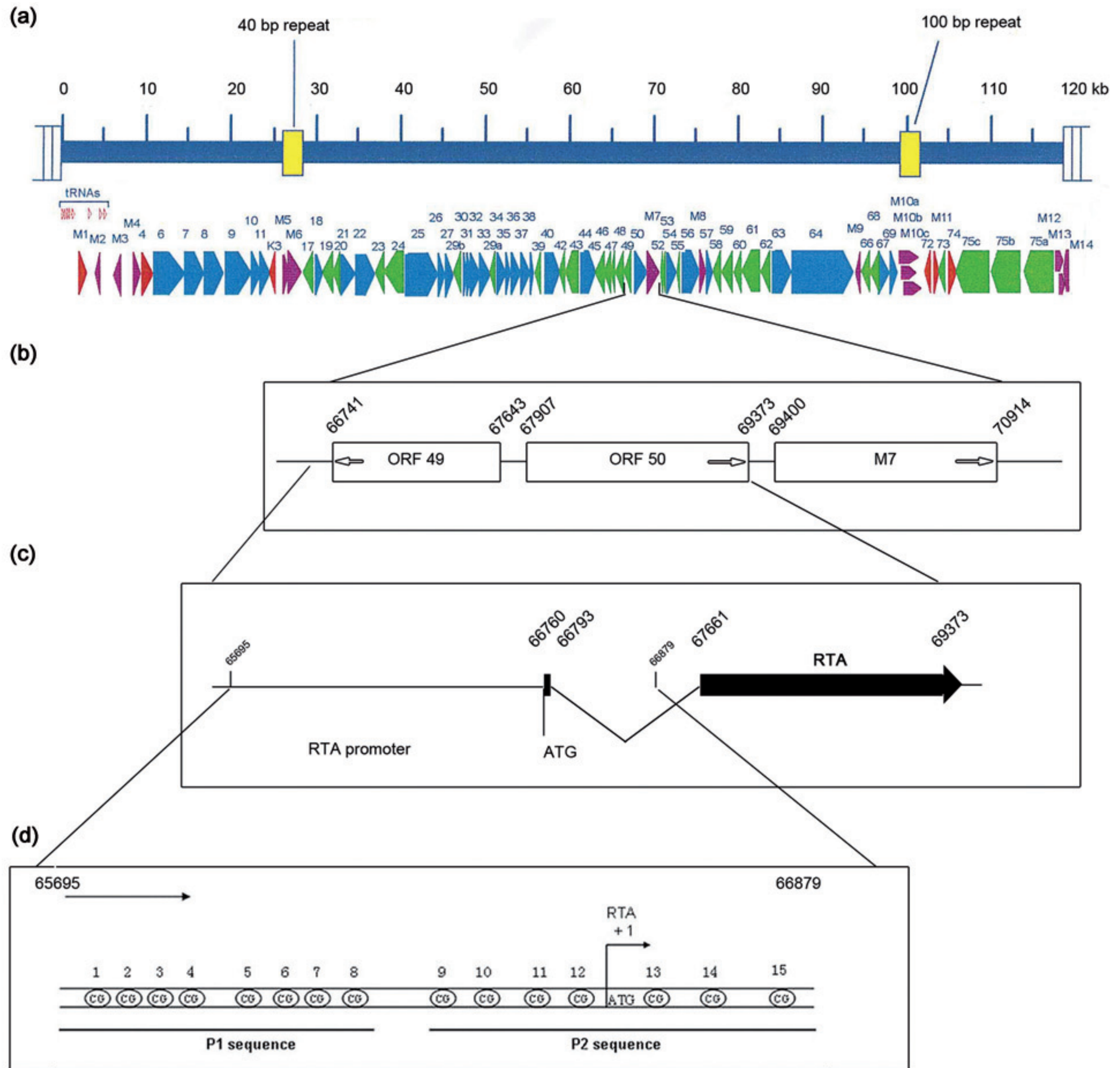

Fig. 1

Schematic diagram of the *Rta*/ORF50 MHV-68

(a) Schematic diagram of the MHV-68 genome and genomic location of ORF50. The nucleotide numbers are assigned on the basis of the MHV-68 sequence (Acc. No. U97553) published in the National Center for Biotechnology Information database (by Virgin *et al.*, 1997). (b) Detail view of the ORF49, ORF50 and M7 genomic region. (c) Scheme of *Rta* transcription region. (d) Detail view of the *Rta* promoter region (by Yang *et al.*, 2009).

excision of the intervening intron results in a single, major *Rta* transcript, but also many bicistronic alternatively spliced *Rta*-containing transcripts can be presented in infected cell. Despite the fact, that ORF50 genes of gammaherpesviruses are highly conserved, the amino acid similarity between KSHV and rhesus monkey rhadinovirus (RRV) *Rta* is 55%, between KSHV and MHV-68 *Rta* is 43%, and between RRV and MHV-68 *Rta* is 47% (Damania *et al.*, 2004). However, these differences do not affect the function of *Rta* protein. Therefore, we considered the possibility that murine gammaherpesvirus strains could possess the amino acid diversity in *Rta* protein. In present study we identified the nucleotide sequence of the ORF50 gene (66760–69373nt) and its proximal promoter region (65695 –66879nt) of MHV-72 and MHV-4556 (Fig. 1c).

Sequences obtained from pJET1.2 recombinants were assembled into a contiguous sequence representing the

region of the ORF50 and its promoter. Short sequence, approximately 640 nt, on the 3' end, which could not be covered by any combination of primers, was verified by the sequencing of the whole genomes of MHV-72 and MHV-4556 by Next-generation sequencing (data not published yet). The sequence analysis showed that the nucleotide sequences of ORF50 gene and its proximal promoter region of MHV-72 and MHV-4556 are identical to that of MHV-68.

However, the regulation of gene expression not only depends on nucleotide sequence of promoter in conjunction with activators and suppressors of gene expression but is also spatially and temporally regulated by epigenetic modifications. These epigenetic mechanisms include DNA methylation, remodelling of nucleosomes, expression of small regulatory RNAs and posttranslational modifications of histone proteins (Bird, 2007). Relatively well characterized chromatin modifications, DNA methylation and histone acetylation, are important for gene transcription regulation, cell development, and also tumorigenesis (Feinberg and Tycko, 2004; Esteller, 2008; Jones and Baylin, 2007). DNA methylation occurs at the 5' position of the cytosine ring within CpG dinucleotides via addition of a methyl group to create a 5-methylcytosine. This process is catalysed by several DNA methyltransferases in eukaryotic cells. Acetylation of histones promotes gene transcription by relaxing chromatin structure and facilitating access of the transcription machinery to DNA, whereas histone deacetylation promotes transcriptional repression by condensation of chromatin structure (Van Opdenbosch *et al.*, 2012). To date, 18 cellular histone deacetylases (HDACs) providing deacetylation of histones are known. Their negative regulators, inhibitors of HDACs, inhibit deacetylation of histones resulting in histones acetylation and thus gene transcription (Damania *et al.,* 2004).

Histones associated with virus genome are important for establishment and maintaining a latent circular viral episome during gammaherpesvirus latency. In addition, transcription from episomal DNA is epigenetically regulated and the gene expression is silenced except for a small number of genes responsible for the maintenance of the episome in the host cells (Eshleman *et al.,* 2011; Djerbi *et al.,* 1999; Ballestas *et al.,* 1999). In previous experiments it was shown that HDAC3 complex is recruited to the *Rta* promoter of MHV-68 and HDACs 1, 5, and 7 are recruited to the *Rta* promoter of KSHV to suppress gene transcription during latency. However, after treatment with HDAC inhibitors, acetylated histones can be found on gammaherpesvirus lytic switch gene promoters (Goodwin *et al.*, 2010). An example can be the treatment with TSA, leading to the removal of HDAC3 from the *Rta* promoter, up-regulation of histone H3 and H4 acetylation level and MHV-68 reactivation. Reactivation is accompanied by passive demethylation at the *Rta* promoter. These findings indicate that histone acetylation induced by TSA, but not DNA demethylation, is sufficient for efficient reactivation of MHV-68 from latency (Yang *et al.*, 2009).

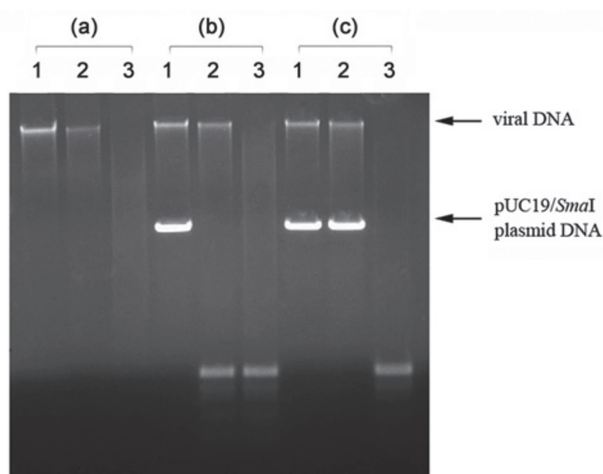

Fig. 2

COBRA analysis of MHV-72 DNA methylation status during late latency (35 d.p.i.) in spleen

(a) MHV-72 DNA from spleen of mice (35 d.p.i.): 1 – non-digested, 2 – digested with *Hpa*II, 3 – digested with *Msp*I; (b) MHV-72 DNA from spleen of mice (35 d.p.i.) mixed with non-methylated pUC19/*Sma*I plasmid DNA: 1 – non-digested, 2 – digested with *Hpa*II, 3 –digested with *Msp*I; (c) MHV-72 DNA from spleen of mice (35 d.p.i.) mixed with methylated pUC19/*Sma*I plasmid DNA: 1 – non-digested, 2 – digested with *Hpa*II, 3 – digested with *Msp*I.

These facts lead us to hypothesise that the strain-dependent reactivation ability (Lopušná *et al.*, unpublished data) may be associated with distinct epigenetic modification of *Rta* promoter, including its methylation status and susceptibility on demethylation enzymes, although we identified that the nucleotide sequences of the ORF50 and its promoter are identical for all three strains tested – MHV-68, MHV-72 and MHV-4556.

We investigated the methylation status of MHV-72 and MHV-4556 DNA during different stages of viral life cycle *in vitro* and *in vivo*. For COBRA analysis we used: 1. DNA isolated from BHK-21 cells infected with MHV-4556 (48 hours post infection) – lytic replication *in vitro*; 2. DNA isolated from the spleen of mice at 14 d.p.i. with MHV-4556 and MHV-72 – early latency *in vivo*; 3. DNA isolated from the spleen of mice at 35 d.p.i. with MHV-4556 and MHV-72 – late latency *in vivo*; 4. DNA isolated from the spleen of mice at 35 d.p.i. with MHV-4556 and MHV-72, after *ex vivo* reactivation induced by TSA – *ex vivo* reactivation; 5. DNA isolated from S11E cell line – natural reactivation. In this cell line MHV-68 occurs in the latent state with a low spontaneous reactivation frequency. We used The Thermo Scientific™ EpiJET™ DNA methylation analysis kit (*Msp*I/*Hpa*II), with a pair of restriction enzymes with different sensitivity to

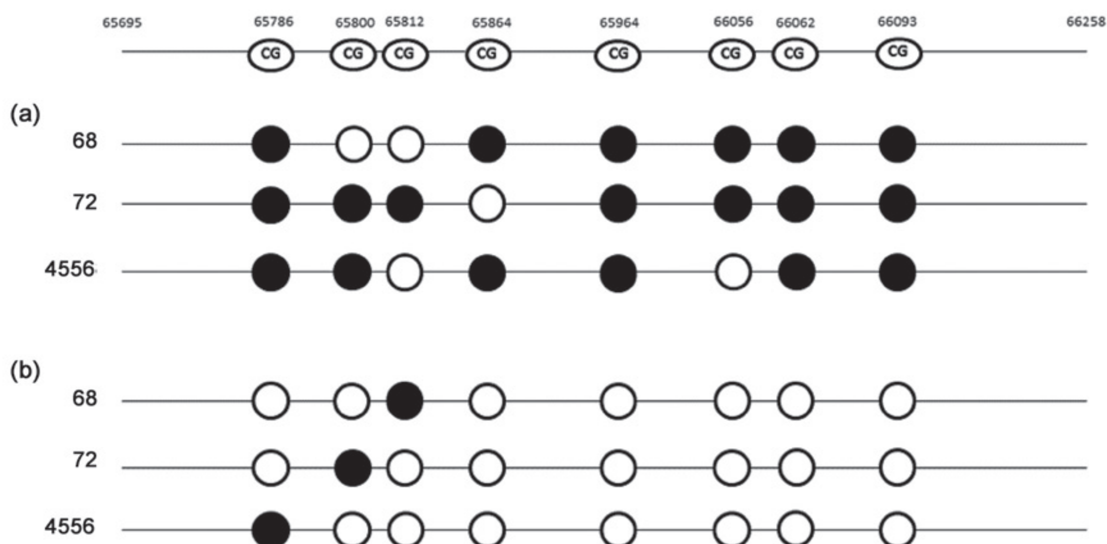

Fig. 3

Bisulfite genomic sequencing (BGS) analysis of the CpG sites at the distal region of *Rta* promoter of MHV-68, MHV-72 and MHV-4556
The distal region (65695–66258nt) of the *Rta* promoter and localization of 8 CpG sites in it. (a) Methylation status of the CpG sites during late latency of MHV-68, MHV-72 and MHV-4556 in spleen at 35 d.p.i. (b) Methylation status of the CpG sites during *in vivo* reactivation of MHV-68, MHV-72 and MHV-4556 induced by TSA (2.4 mg/kg) in spleen at 35 d.p.i. Solid circles indicate methylated CpG, open circles indicate demethylated CpG.

methylated DNA. When the CpG site is methylated, DNA cleavage with *Hpa*II is blocked, although the cleavage with *Msp*I is not affected.

We have found that the DNA isolated from BHK-21 cells infected with MHV-4556 (1) could be digested by *Hpa*II, suggesting that DNA is mostly unmethylated during lytic replication *in vitro* (48 hours post infection). DNA isolated from spleen of mice infected with MHV-4556 and MHV-72 was resistant to *Hpa*II digestion (Fig. 2, line a2, b2, c2), indicating mostly methylated status of DNA during the early latency (2) and the late latency in spleen (3). During the *ex vivo* reactivation induced by TSA (4) and also during the natural reactivation in S11E cell line (5), the DNA showed the presence of both, methylated and unmethylated cytosines, since it was partially resistant to *Hpa*II digestion (data not shown).

With respect to the fact, that the *ex vivo* induced reactivation does not include the complexity of *in vivo* processes and virus/host interactions, we investigated virus reactivation from latency *in vivo,* which was induced by TSA administered as a subcutaneous injection to mice at 35 d.p.i. with MHV-68, MHV-72 or MHV-4556. For detailed examination of the methylation/demethylation status of MHV-4556 and MHV-72 *Rta* promoters during latency and after *in vivo* induced reactivation, we performed the bisulfite sequencing analysis (BGS) consisting of the bisulfite conversion of unmethylated cytosines, the methylation specific PCR (MSP) and the sequencing of amplified *Rta* fragments. The 1kbp MHV-68, MHV-72, and MHV-4556 *Rta* promoter region was divided into two fragments, defined as P1 and P2, which include a total of 15 CpG sites (Fig. 1d). The distal region of *Rta* promoter, the fragment P1 (65695–66258 nt), consists of 8 CpG sites, the proximal region, the fragment P2 (66355–66879 nt), consists of 7 CpG sites, which surround the transcription start of the *Rta* (66760 nt). We found that during latency (35 d.p.i.), most of the CpG sites in the P1 fragment of *Rta* promoter were methylated (Fig. 3a) and all of the CpG sites in the P2 fragment were methylated (data not shown). However, no significant differences of methylation status of *Rta* promoter region between the strain MHV-68, MHV-72 and MHV-4556 were detected in late latency *in vivo*. TSA treatment during *in vivo* induced reactivation (for 5 hr) led to the effective demethylation of most of the CpG sites in the P1 fragment of *Rta* promoter (Fig. 3b) and all CpG sites in the P2 fragment (data not shown).

Taken together, these data indicate that the methylation patterns of the *Rta* promoter of MHV-68, MHV-72 and MHV-4556 were remarkably uniform during latency as well as the demethylation patterns of the *Rta* promoter of all three strains during *in vivo* reactivation induced by TSA. Furthermore, we can conclude that the sensitivity of MHV-72 and MHV-4556 to HDAC inhibitor Trichostatin A is similar within all three strains, MHV-68, MHV-72 and MHV-4556.

First evaluation of the *Rta* methylation status associated with MHV-68 infection *in vitro* and *in vivo* was done by Yang *et al.* (2009). Similarly to our results, they found that the viral *Rta*

promoter region of MHV-68 was highly methylated during latency *in vitro* and *in vivo*, whereas the *Rta* promoter was mostly unmethylated during *de novo, in vitro* or *in vivo* infection.

Summarizing, we did not find any differences between studied murine gammaherpesvirus strains – MHV-68, MHV-72 and MHV-4556, which could be associated with the replication transcription activator, *Rta* (ORF50), and its epigenetic regulation during lytic infection, latency and also *de novo* infection after *ex vivo* and *in vivo* induced reactivation. Moreover, we confirmed that the treatment with TSA successfully induced demethylation of the *Rta* promoter regions of all three studied strains, and this occurred by passive mechanism (Yang *et al.*, 2009).

Therefore, the unique *in vivo* pathogenetic properties of MHV-72 and MHV-4556, including mainly the reduced capacity to reactivate from latency followed by low amounts of genome copies of *de novo* assembled virus, could be related to another gene or genes, in which they differ from prototype strain MHV-68.

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